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Post Protein-Binding Reactivity and Modifications of the *fac*-$[Re(CO)_3]^+$ Core.

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Supporting Information Placeholder

ABSTRACT: The reactivity of the $[Re(CO)_3(H_2O)_2]^+$ complex coordinated to the His15 residue of HEW lysozyme is described. In the fully metallated protein (**Lys-1**), the Re ion retains its reactivity only towards selected ligands while others induce a ligand-mediated de-metallation of the enzyme. It is further shown that some of the complexes which may be "engineered" *on* the lysozyme do not react with the free protein even if present in solution in excess. The formation of stable metal adducts starting from **Lys-1** was confirmed by X-ray crystallography.

Chemical hybridization of proteins with non-native metal fragments constitutes an active field of research. Some groups have brilliantly directed their efforts towards the development of artificial enzymes[1-12] while others have exploited the exogenous metal complexes in the study of long-rage electron transfer reactions or photo-induced relaxation processes.[13-16] In the case of metal ions which find applications in medicinal chemistry, the interaction of polypeptides with metal drugs is mainly investigated in order to elucidate the fate of the drug within the blood stream or in cellular compartments.[17]

Several studies have now made available structural information of the interactions of, mainly, Pt and Ru-based anticancer agents with cellular proteins.[18-21] In contrast, the reactivity of the protein-bound complexes has received far less attention. It is of interest, in our opinion, not only to determine the location, the structural modifications, the strength of binding and the reversibility of protein-metal complex interactions, but also to understand how the nature of the chiral protein environment might influence the chemistry of the metal complex.

This latter question has been explored only marginally. However, possible post protein-binding reactions of e.g. metallodrugs, might play an important role in determining the biochemical basis of the systemic toxicity of the drugs or play a role in their therapeutic effects. Elucidating the chemistry of protein-bound metal species might also offer an elegant alternative to the seleno-methionine dependant multiwavelength anomalous diffraction (MAD) method for solving the so-called "phase-problem".[22]

Herein we show (see Figure 1) that the chemistry of the lysozyme-bound *fac*-$[Re(CO)_3]^+$ core (**Lys-1**) is affected by the nature of the chiral protein environment when compared to the solvated metal ion (i.e. *fac*-$[Re(CO)_3(OH_2)_3]^+$ (**1**)). The Re ion on the fully metallated enzyme **Lys-1** retains its reactivity towards selected mono and bidentate ligands while molecules bearing primary amines induced de-metallation of **Lys-1**.

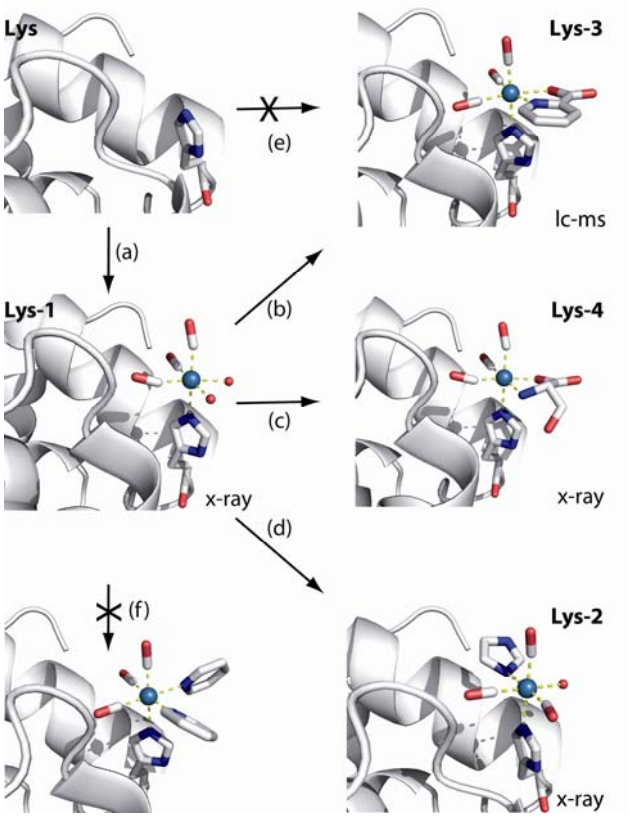

Figure 1. Reaction scheme leading to the species described in this contribution. (a) complete metallation of HEW lysozyme (**Lys**) with the *fac*-$[Re(CO)_3(OH_2)_3]^+$ complex (**1**). (b), (c) and (d) derivatizazion of **Lys-1** with pyridine-2-carboxylic acid (pa), L-serine and imidazole yielding **Lys-3**, **Lys-4** and **Lys-2** respectively. (e) unsuccessful synthesis of **Lys-3** via reaction of **Lys** with the *fac*-$[Re(CO)_3(pa)(OH_2)]$ complex. (f) reaction of **Lys-1** with pyridine showed no evidence of ligand exchange at the metal core.

The x-ray structure of the lysozyme-bound $[Re(CO)_3(OH_2)_2]^+$ complex (metal ion occupancy modeled to 60%) was recently communicated by Ziegler and co-workers.[24,25] We found that complete metallation of the enzyme could be attained by allowing **Lys** to react with an excess of **1** (>20eq.) for 7 days at room tempera-

ture. Mass spectrometry (Supporting Information (SI)) clearly showed the formation of the corresponding adduct (**Lys-1**) in quantitative yield. Chromatographic

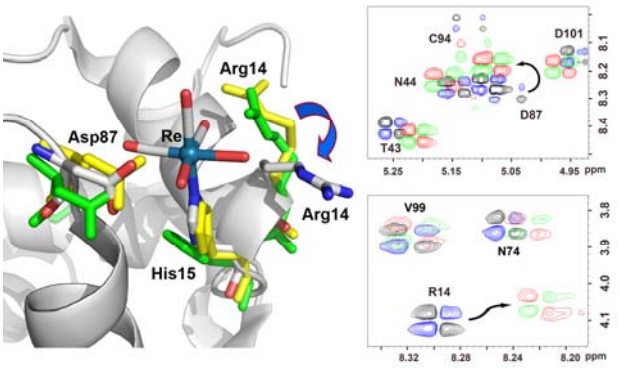

Figure 2. Left: comparison of the X-ray structure of **Lys-1** (gray, PDB entry 3KAM), a high resolution X-ray structure of **Lys** (yellow, PDB entry 2VB1) and the NMR structure of **Lys** (green, PDB entry 1E8L). Right: selected fingerprint regions of phase-sensitive 500-MHz COSY spectrum of non-exchanged **Lys** (blue-black) and **Lys-1** (red-green). Residues assignments are as previously reported.[23]

purification of **Lys-1** allowed isolating the protein in ca. 80% yield with no apparent loss of the metal ion (typically on a 50 mg scale, SI). CD studies and enzymatic assays on the rate of lysis of *Micrococcus lysodeikticus* showed that the chromatographic purification did not affect enzymatic activity or folding of **Lys-1** (SI).[26] The metallated protein thus obtained could also be crystallized but we found no significant differences with the reported structure with the exception that Re occupancy could be modelled to a value >80%.

500-MHz COSY spectrum of non-exchanged **Lys** and **Lys-1** are compared in Figure 2. Coordination of **1** to His15 induced a downfield shift of the resonances of the residue which was lost under the presaturated water signal (SI). The most significant resonance shift was noticed for Arg14 (labelled R14 in Figure 2), an observation which may be rationalized by comparing the x-ray structure of **Lys-1** with the NMR solution structure of the free enzyme (Figure 2). The resonance changes of Arg14 are consistent with the rearrangement of the residue required to lift the steric hindrance at the binding site. Overall COSY cross-peak signals of **Lys-1** retained the same pattern as the one observed for the free lysozyme (SI).[23]

The reactivity of **Lys-1** was studied in water by exposing the metalloprotein to different mono and bidentate ligands. Preliminary studies of these interactions were always followed by LC-MS. Under our experimental conditions, MS analysis of the **Lys-** and **Lys-1**-derived species always showed a distinct pattern of signals (typically 4) corresponding to the +8 to the +11 ions of the enzymes (SI). Changes in the position of the signal patterns offered an immediate indication of the type of interactions which resulted from the reactions of **Lys-1** (SI).

Reaction of **Lys-1** with imidazole (im, 10 eq. 12h, RT) gave the corresponding *fac*-$[Re(CO)_3(His15)(im)(OH_2)]^+$ adduct (**Lys-2**) in good yield. The coordination of a second imidazole was never observed. The X-ray structure of **Lys-2** is shown in Figure 3 (*vide infra*). Under similar conditions, no reaction was observed when **Lys-1** was dissolved into an aqueous solution of pyridine (py). On the other hand, reactions of the solvated metal ion **1** with im gave a mixture of products (i.e. mono-, bis- and tris- substituted complexes), while with py the well defined *fac*-$[Re(CO)_3(py)_2(OH_2)]^+$ complex.[27]

The fundamental reasons underlying these differences were not clear at this point. We speculated that hydrogen-bonding interactions between the incoming ligands and the amino acid residues in close proximity to the rhenium binding site may play an important role in determining the reactivity of the metal ion. To test this hypothesis, **Lys-1** was reacted with pyridine-2-carboxylic acid (pa) and 2-methylamine-pyridine (pn) under conditions described above. Reaction of **Lys-1** with pa gave the corresponding *fac*-$[Re(CO)_3(His15)(pa)]$ adduct (**Lys-3**) (SI) but when exposed to an aqueous solution of pn, **Lys-1** was readily de-metallated resulting in a mixture of free **Lys** and the *fac*-$[Re(CO)_3(pn)(OH_2)]^+$ complex. This type of ligand-mediated de-metallation of **Lys-1** was further observed in the reactions of the metalloprotein with other bidentate ligands bearing primary amines (e.g. ethylenediamine). Crystallization of samples of **Lys-3**, obtained after chromatographic purification, was attempted under a wide variety of conditions, but no diffracting crystals could be obtained.

In order to get insights into the possible interactions of **Lys-1** with more biologically relevant molecules, the metalloprotein was also reacted with amino acids. We focused our attention on L-serine (L-ser). Under conditions similar to those described for the synthesis of the im conjugate, **Lys-1** reacted with L-ser to give the corresponding *fac*-$[Re(CO)_3(His15)(L\text{-}ser)]$ adduct (**Lys-4**) in ca. 80% yield. Partial loss of the metal fragment was observed during the reaction but no further de-metallation was detected after chromatographic purification.

Crystals of **Lys-2** and **Lys-4** suitable for X-ray diffraction analysis were obtained by the hanging-drop method (SI). Figure 3 show the overall structure of **Lys-2** and a detailed view of the metal binding site of **Lys-4**. A similar detailed view of the binding site of **Lys-2** is given in SI. It should be mentioned that in the case of **Lys-2**, the difference map contoured at the metal binding site does not entirely show the imidazole ring. However, MS analysis of the single crystal employed in the X-ray diffraction analysis unequivocally points to the presence of imidazole in the rhenium coordination sphere (SI).

Lys-4 crystallized in a typical tetragonal lysozyme cell (space group P43212, PDB accession code 3QNG). Two molecules of **Lys-2** were found in the asymmetric unit of the orthorhombic cell (space group P212121, PDB accession code 3QE8). The structures were solved by molecular replacement using a **Lys** model (PDB entry 1IEE) and the restraints for the rhenium complexes were taken from the corresponding small molecule structures.[28,29] In both cases, the rhenium complexes were refined with 80% occupancy. The crystals of **Lys-2** and **Lys-4** were diffracting till 1.55 and 1.49 Å respectively (SI).

It is interesting to point out that in solid state structure of **Lys-4** a single *fac*-$[Re(CO)_3(His15)(L\text{-}ser)]$ diastereomer is observed. In the structure, the OH group of L-ser is H-bonded to the threonine residue 89 (SI). It is possible that one of the two diastereomeric forms of **Lys-4** was selectively crystallized or alternatively the hydrogen-bonding interaction is responsible for the binding specifici-

ty of L-ser. We tend to favour this second hypothesis as diastereomerically pure samples of *fac*-[Re(CO)$_3$(L-ser)(X)] (where X = im type ligand) are known to rapidly epimerize at the rhenium center in aqueous solutions.[29]

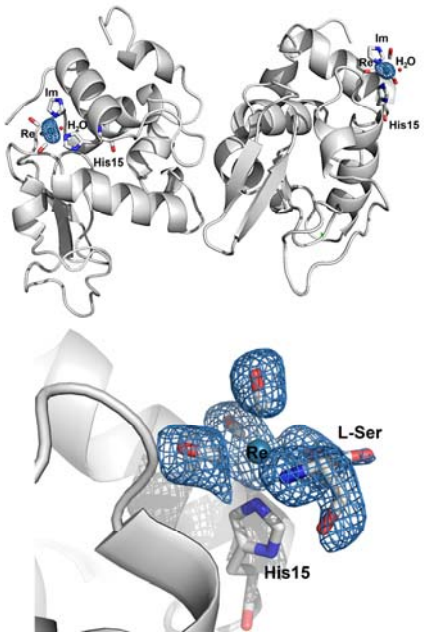

Figure 3. Top: overall structure of **Lys-2** in the asymmetric unit (anomalous electron density contoured at 4.0 σ). Bottom: detailed view of the metal binding site of **Lys-4** (difference electron density map contoured at 2.7 σ, SI). The pictures were prepared using the Pymol software.[30]

In summary, a study of the post protein-binding reactivity of the lysozyme-bound *fac*-[Re(CO)$_3$]$^+$ core was presented. The study corroborated the assumption that the nature of the chiral protein environment might affect the chemistry of a metal complex on a protein surface. Our results led us to hypothesize that hydrogen-bonding interactions between the incoming ligands and the amino acid residues in close proximity to the metal-binding site may play an important role in determining the reactivity and the specificity of binding of the metal ion.

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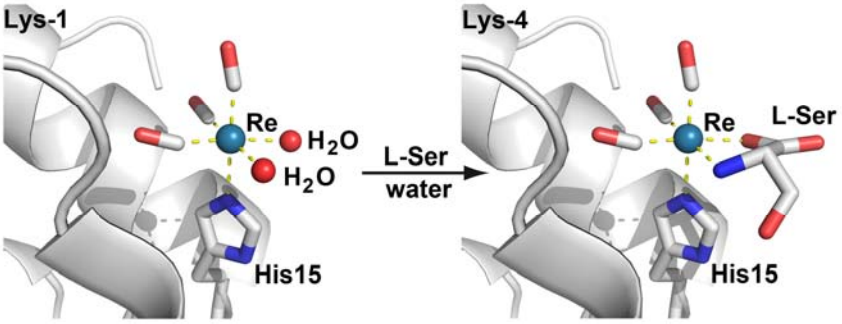

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